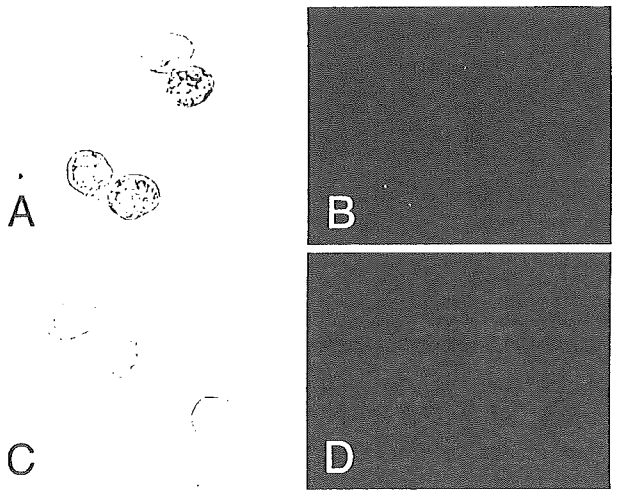

FIG. 3. Immunocytochemistry of *E. histolytica* with a fusion protein of human Fab CP33 and alkaline phosphatase. Paraformaldehyde-fixed trophozoites were treated with the fusion protein (A and B) and then with the substrate Vector red. As the controls (C and D), trophozoites were treated with a supernatant of *E. coli* lysates (vector control). (A and C) Bright-field microscopy; (B and D) fluorescence microscopy with a green filter. Magnification, ×360.

cently for application in immunoprophylaxis, or the treatment of infectious diseases. Although such human antibodies would also be useful for diagnostic purposes, one of the disadvantages of the use of human antibodies for the detection of pathogens in human samples might be reactivity of endogenous immunoglobulins with the secondary anti-human antibodies used in indirect methods. Therefore, direct labeling of the human antibody with enzymes is needed to reduce nonspecific binding of the second antibody. It was reported recently that immunoglobulin genes derived from murine hybridoma cells could be expressed in *E. coli* as fusion protein Fab-PhoA (7, 25) or scFv-PhoA (5, 7, 13, 17). The present study demonstrates that the bacterial expression of a human MAb-PhoA conjugate specific for *E. histolytica* is also possible. In addition to the advantage of using the antibody to detect the *E. histolytica* antigen without the need for chemically conjugated secondary antibodies, there is no requirement for experimental animals or reagents and equipment for the culture and cryopreservation of hybridoma cells. Accordingly, the use of this human recombinant antibody also provides an economic benefit.

The antigen recognized with CP33 was the heavy subunit of the galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin of *E. histolytica* (23). It is well known that this lectin molecule is suitable as a target antigen for the detection of *E. histolytica* in fecal and serum samples (1, 2, 11, 12). In conclusion, we propose here that the human Fab-PhoA fusion protein can be used in the diagnosis of amebiasis.

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VH3 Gene Usage in Neutralizing Human Antibodies Specific for the *Entamoeba histolytica* Gal/GalNAc Lectin Heavy Subunit

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A combinatorial human immunoglobulin gene library was constructed from peripheral lymphocytes of an asymptomatic *Entamoeba histolytica* cyst passer and screened for the production of Fab antibody to the parasite. One of the Fab clones, CP33, recognized the 260-kDa galactose- and *N*-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin of *E. histolytica*. By shuffling the heavy and light chains of CP33 with the heavy and light chains of two libraries derived from the cyst passer and a liver abscess patient, 18 additional clones were obtained. Sequence analysis of the heavy-chain genes, including CP33-H, revealed that all the nearest V-segment germ lines belonged to the VH3 family (VH3-21, VH3-30, VH3-48, and VH3-53), but the levels of homology were only 85 to 95%. The closest D-segment germ line was D2-2 or D6-6, and for the J-segment the closest germ line was JH4b or JH6b. On the other hand, all the light-chain genes, including CP33-L, belonged to the Vκ1 family, in which the closest Vκ germ line gene was 02/012 or L5, with the Jκ1, Jκ2, Jκ4, or Jκ5 segment. CP33 and three other Fabs obtained by light-chain shuffling were purified and analyzed further. All of these Fabs recognized the cysteine-rich domain of the 170-kDa heavy subunit of the Gal/GalNAc lectin. Preincubation of *E. histolytica* trophozoites with these Fabs significantly inhibited amebic adherence to Chinese hamster ovary cells and also inhibited erythrophagocytosis. The ability of the neutralizing antibodies to block erythrophagocytosis for the first time implicates the lectin in phagocytosis and VH3 antibodies in defense against parasitic infections. These results demonstrate the utility of a combinatorial human immunoglobulin gene library for identifying and characterizing neutralizing antibodies from humans with amebiasis.

Worldwide, the intestinal protozoan parasite *Entamoeba histolytica* causes an estimated 50 million cases of amebic colitis and liver abscess annually, resulting in up to 110,000 deaths (48). The ability of *E. histolytica* trophozoites to invade the colon and other tissues depends on several pathogenic factors. One of the most important factors is the galactose (Gal)- and *N*-acetyl-D-galactosamine (GalNAc)-inhibitable cell surface lectin of the ameba. The lectin mediates adherence of trophozoites to human colonic mucins, colonic epithelial cells, neutrophils, and erythrocytes (4, 7, 18, 24, 37, 38). The Gal/GalNAc lectin is also important in the cytolytic event that follows adherence. The amebic lectin is a 260-kDa hetrodimeric glycoprotein composed of 170-kDa heavy and 35- or 31-kDa light subunits (32). In addition, a 150-kDa intermediate subunit of the lectin also contributes to adherence (8, 12). Immunization of experimental animals with these lectin subunits provides protection from liver abscess formation (10, 25, 27, 34, 50). Such protection is also observed after passive immunization with a mouse monoclonal antibody to the intermediate subunit lectin (11).

Recently, it has been shown that *E. histolytica*-specific human monoclonal antibody Fab fragments can be prepared from the peripheral lymphocytes of a patient with an amebic liver abscess (9, 42). One of the clones which recognized the 260-kDa Gal/GalNAc lectin inhibited the adherence of trophozoites to mammalian cells (9). Successful cloning of the active fragments might be due to the patient's high antibody titer to *E. histolytica*.

There are also clinically asymptomatic individuals, which pass *E. histolytica* cysts in their stools. Most of the cyst passers have a positive serology for the ameba, although their antibody titers are low (44). In such cyst passers, there may be protective antibodies that block invasion of trophozoites into tissues. However, little is known about the immune response to *E. histolytica* in asymptomatic cyst passers. Therefore, molecular analysis of the immune response to the amebic lectin is important for understanding protective immunity and for vaccine development. We report here molecular cloning of immunoglobulins specific for the *E. histolytica* Gal/GalNAc lectin. The clones were derived from the peripheral lymphocytes of an asymptomatic cyst passer. We also report possible recombination and bacterial expression of antibody genes from both aymptomatic and symptomatic individuals.

MATERIALS AND METHODS

Cultivation of parasites. Trophozoites of 10 strains of *E. histolytica* (HM-1:IMSS, HK-9, 200:NIH, HB-301:NIH, H-302:NIH, H-303:NIH, DKB, C-3-2-1, SAW1627, and SAW755CR) were axenically grown in BI-S-33 medium (16). Trophozoites of *Entamoeba dispar* SAW1734RclAR were cultured monoxenically with *Pseudomonas aeruginosa* in BCSI-S medium (22). Trophozoites were washed three times with ice-cold 10 mM phosphate-buffered saline (PBS) (pH 7.4) before they were used.

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Construction of immunoglobulin gene library. Ten milliliters of peripheral blood was collected from the asymptomatic cyst passer. Cysts detected in the feces had been identified specifically as *E. histolytica* by PCR. The serum was positive for *E. histolytica* with a titer of 1:64 (borderline positive), as determined by an indirect fluorescent-antibody (IFA) test. Lymphocytes were separated from the blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. RNA was isolated from the lymphocytes with an RNeasy total RNA purification kit (QIAGEN GmbH, Hilden, Germany). Reverse transcriptase PCR was performed with a GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's instructions. An oligo$(dT)_{16}$ primer was used for cDNA synthesis. Genes encoding the κ and λ light chains and the Fd region of the γ heavy chain were amplified as previously described (42). Thirty-five cycles of PCR were performed as follows: denaturation at 94°C for 1 min (5 min in cycle 1), annealing at 50°C for 2 min, and polymerization at 72°C for 3 min (10 min in cycle 35). The light-chain genes were first ligated with an expression vector, pFab1-His2 (46), and then introduced into XL1-Blue Epicurian Coli (Stratagene, La Jolla, Calif.). The vector with inserts was selected, and then the Fd heavy-chain genes were ligated into the vector and introduced into the bacteria.

Expression of immunoglobulin genes and screening of clones producing anti-*E. histolytica* antibodies. Screening of positive clones was performed as previously described (9). The expression vector containing light-chain and Fd heavy-chain genes was introduced into competent *Escherichiacoli* JM109. Approximately 10^3 to 3×10^3 colonies per 90-mm plate were grown on Luria-Bertani agar plates containing 50 μg of ampicillin per ml at 37°C. Colonies were transferred to nitrocellulose filters and then incubated on fresh plates containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and ampicillin at 30°C for 6 h. Each filter was treated with chloroform vapor and then incubated with lysis buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 1.5% bovine serum albumin, 1 μg of DNase per ml, 40 μg of lysozyme per ml) overnight. After washing with PBS containing 0.05% Tween 20 (PBS-Tween), the filter was incubated in PBS-Tween containing 5% skim milk and then with 400 μg of soluble *E. histolytica* antigen prepared from trophozoites of the HM-1:IMSS strain per ml. After washing, the filter was incubated with horseradish peroxidase (HRP)-conjugated antibody purified from the plasma of a patient with an amebic liver abscess. The filter was washed and then developed with a Konica immunostaining kit (HRP-1000; Konica Co., Tokyo, Japan). Positive clones were identified in original plates and then cultured in 5 ml of super broth (30 g of tryptone per liter, 20 g of yeast extract per liter, 10 g of morpholinepropanesulfonic acid [MOPS] per liter; pH 7) containing ampicillin until an optical density at 600 nm of 0.6 to 0.7 was obtained. IPTG, at a final concentration of 100 μM, was added to the bacterial cultures, which were then incubated at 30°C for 14 h. The bacteria were pelleted by centrifugation, suspended in 250 μl of PBS containing 1 mM phenylmethylsulfonyl fluoride, and then sonicated. The lysates were centrifuged at $12,000 \times g$ for 10 min, and the supernatant was subjected to a second screening by performing an IFA test with intact trophozoites.

IFA test. The IFA test, with live intact trophozoites (45) or formalin-fixed trophozoites smeared on glass slides (43), was performed as previously described. Fluorescein isothiocyanate-conjugated goat immunoglobulin G (IgG) to human IgG Fab (Organon Teknica Co., Durham, N.C.) was used as the second antibody.

Shuffling of light- and heavy-chain genes and rescreening. The light-chain gene of the positive clone was replaced with light-chain genes from the library constructed from the asymptomatic cyst passer. The light-chain-shuffled plasmid containing the cloned Fd heavy chain was introduced into the bacteria and then screened again. The Fd heavy chain of the positive clone was also replaced with the library's heavy-chain genes and screened again. Another immunoglobulin library, prepared from a patient with an amebic liver abscess (9), was also used as a source of light- and heavy-chain genes. Positive clones were subjected to a second screening by the IFA test. Light- and heavy-chain genes of clones positive in the second screening were amplified by PCR, digested with *Mbo*I, *Rsa*I, *Dra*I, *Hin*fI, and *Hae*III and with *Mbo*I, *Rsa*I, and *Alu*I, respectively, and then compared by performing electrophoresis in an agarose gel.

ELISA. The soluble antigen derived from *E. histolytica* trophozoites, which was used for screening clones, was also used for an enzyme-linked immunosorbent assay (ELISA). The wells of the ELISA plates, containing 5 μg of antigen diluted with 50 mM sodium bicarbonate buffer (pH 9.6), were incubated overnight at 4°C. The plates were washed with PBS-Tween and then treated with PBS containing 3% skim milk for 1 h. For use in the ELISA, positive clones of bacteria were cultured in 10 ml of medium, and then 0.5 ml of the resultant supernatant was prepared as described above. One hundred microliters of the supernatant was added to the wells and incubated for 1 h at room temperature. After the wells were washed, they were incubated with 100 μl of HRP-conjugated sheep antibody to human IgG $F(ab')_2$ (ICN Pharmaceuticals, Aurora, Ohio) for 1 h at room temperature. After they were washed with PBS-Tween, they were incubated with 200 μl of a substrate solution (0.4 mg of *o*-phenylenediamine per ml in citric acid-phosphate buffer [pH 5.0] containing 0.001% hydrogen peroxide). After 30 min of incubation, the reaction was stopped by addition of 50 μl of 2.5 M H_2SO_4, and the optical density at 490 nm was determined with a model 550 microplate reader (Bio-Rad, Hercules, Calif.).

DNA sequencing. Cloned light-chain genes and Fd heavy-chain genes were recloned into sequencing vectors. Cycle sequencing in both directions was performed with Thermo Sequenase (Amersham Life Science, Cleavland, Ohio) by using M13 primers. Reactions were performed with a model 4000L automated DNA sequencer (LI-COR, Lincoln, Nebr.).

Purification of Fabs. Positive clones were cultured in 1 liter of medium. Twenty milliliters of each resultant supernatant, prepared as described above, was filtered through 0.22-μm-pore-size filters and used for purification of Fab fragments. Purification of Fabs was performed by affinity chromatography by using His·Bind Resin (Novagen, Madison, Wis.) according to the manufacturer's instructions.

SDS-PAGE and Western immunoblot analysis. Purified Fab fragments and trophozoites of *E. histolytica* HM-1:IMSS in PBS were solubilized with an equal volume of the sample buffer (23) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 2 mM *p*-hydroxymercuriphenyl sulfonic acid, and 4 μM leupeptin for 5 min at 95°C. After centrifugation, the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western immunoblot analysis was performed as previously described (43). HRP-conjugated sheep antibody to human IgG $F(ab')_2$ (ICN Pharmaceuticals) was used as the second antibody. Development was with a Konica HRP-1000 immunostaining kit.

Dot immunoblot analysis. Affinity-purified 260-kDa Gal/GalNAc lectin and the recombinant protein of the cysteine-rich domain in the 170-kDa heavy subunit of the lectin (rLecA), prepared as previously described (28), were used for a dot immunoblot analysis. One microgram of each of these antigens in PBS was spotted onto a nitrocellulose membrane and air dried. Each spot on the membrane was blocked for 30 min with PBS containing 3% skim milk and then incubated with 2 μg of each Fab for 1 h at room temperature. As controls, normal human Fab (OEM Concepts, Toms River, N.J.), antibody purified from the plasma of a patient with an amebic liver abscess, and rabbit antibody to the 260-kDa lectin were used. After the membrane was washed three times with PBS-Tween, it was incubated with HRP-conjugated sheep antibody to human IgG $F(ab')_2$ or HRP-conjugated goat antibody to rabbit IgG (ICN Pharmaceuticals). After washing, each antibody-bound dot was detected with a Konica HRP-1000 immunostaining kit.

Determination of affinity constant. Affinity measurement of purified Fabs by surface plasmon resonance was carried out by using a BIAcore 3000 instrument (Biacore AB, Uppsala, Sweden) according to the general procedure outlined by the manufacturer. Affinity-purified 260-kDa Gal/GalNAc lectin and rLecA were immobilized onto a CM5 chip (Biacore) surface at a low density by amine coupling chemistry. Affinity constants were determined by using the software provided by the manufacturer, BIAevaluation 3.1.

Adherence assay. Adherence of *E. histolytica* to Chinese hamster ovary (CHO) cells was evaluated as previously described (49). Briefly, trophozoites (10^4 cells) of the HM-1:IMSS strain were incubated with 100 μg of each Fab for 1 h at 4°C, washed with ice-cold PBS, and then suspended in Ham's F12 nutrient mixture containing 1% adult bovine serum. As a control, normal human Fab (OEM Concepts) was used. The trophozoites and CHO cells (2×10^5 cells) were suspended in 1 ml of the Ham's F12 nutrient mixture, centrifuged, and then incubated at 4°C for 2 h. After removal of 0.8 ml of supernatant, the pellet was gently vortexed, and the number of trophozoites with at least three adherent CHO cells was determined by examining 300 trophozoites.

Erythrophagocytosis assay. Erythrophagocytosis of *E. histolytica* was assayed as previously described (47). Briefly, trophozoites (10^4 cells) of HM-1:IMSS were exposed to 100 μg of each Fab at 37°C for 30 min and then washed with ice-cold PBS. Normal human Fab (OEM Concepts) was also used as a control. The erythrophagocytosis assay was performed by mixing the Fab-treated trophozoites with 10^6 human erythrocytes and then incubating the preparation at 37°C for 5 min. After lysis of free and adherent erythrocytes by addition of distilled water, the trophozoites were fixed and stained with a 3,3'-diaminobenzidine solution containing hydrogen peroxide. The number of erythrocytes ingested was determined by examining 300 trophozoites.

Nucleotide sequence accession number. The nucleotide sequence data reported here have been deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB095272 to AB095291.

```
                    FR1                CDR1       FR2            CDR2
CP33-H   EVKLMESGGGVVQPGRSLRLSCAASGFRFS TYAIH WVRQAPGKGLEWVA RISHDGSQTHYADSVQG
H-CP1    Q.Q.K.....L.K..G...........T.. DFSMN ............S S.TATSVYLK.....R.
H-CP4    D.Q.Q..................................... ..... ..... .....
H-CP5    ....LQ..---------...V.......TVN SNHMS ............S I.YSA-GI.Y.....K.
H-CP6    ....L....DLL...G...........T.. ...MN ............S Y..SGSGTIY.....K.
H-LA5    ..... ..... ..... .....

                    FR3                      CDR3          FR4
CP33-H   RFGVSRDNSNYTAYVQLNSLRPDDTAVYFCAR AYSSTP---DYGMDV WGQGTAVTVSS
H-CP1    ..TI.....DNSV.L.MTNVSGE.....Y... DGGGKAASGY..... .....T.....
H-CP4    ..... ..... .....---..... .....
H-CP5    ..TI.....EN.LFL.M.....E.....Y... GKY.PS--IG.YF.Y .....L.....
H-CP6    ..TI....AKNSVSL.M....GE.....Y... GPRFEM----APF.Y .....L.....
H-LA5    ..... ..... .....---..... .....
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FIG. 1. Deduced amino acid sequences for genes coding for heavy-chain variable regions of human anti-*E. histolytica* Fab fragments. FR, framework regions. The dashes and dots indicate deletions and identical residues, respectively.

RESULTS

Cloning of recombinant Fab with *E. histolytica*. The combinatorial immunoglobulin gene library constructed from peripheral lymphocytes of an asymptomatic *E. histolytica* cyst passer contained 9.6×10^6 clones. When 6.32×10^4 clones were screened by colony blotting, 6 clones (0.0095%) showed positive signals. In the second screening performed by IFA with intact cells, two clones were positive. Since one of the positive clones, designated CP33, was reactive with the 260-kDa Gal/GalNAc lectin in a preliminary dot immunoblot analysis, this clone was analyzed further.

Chain shuffling of the recombinant Fab. To find other heavy- and light-chain genes which constitute antilectin Fab fragments with light- and heavy-chain genes of CP33, the libraries from the asymptomatic cyst passer (CP library) and the amebic liver abscess patient (LA library) were screened again after shuffling of heavy- and light-chain genes of CP33 with genes from the two libraries. Many positive signals were detected after chain shuffling. When the light-chain gene of CP33 was shuffled, the positive rates in the colony blot screening were 10- or 20-fold higher than those in the shuffling analysis of the heavy-chain genes (1.92 and 0.19%, respectively, for the LA library; 0.96 and 0.047%, respectively, for the CP library). In addition, when the LA library was used as the source of immunoglobulin genes, the positive rates were two- or fourfold higher than those obtained with the CP library (1.92 and 0.96%, respectively, for light-chain shuffling; 0.19 and 0.047%, respectively, for heavy-chain shuffling). All positive clones were secondarily screened with an IFA by using formalin-fixed trophozoites of *E. histolytica* HM-1:IMSS, and then the genes of IFA-positive clones were compared by restriction enzyme digestion. Based on the digestion patterns, 12 clones of the light-chain genes and four clones of the heavy-chain genes were identified as different from each other and also different from the light- and heavy-chain genes of CP33. Clones that showed the same digestion pattern as CP33 but were derived from the LA library were also selected. When more than two clones showed the same digestion pattern, the clone with the strongest IFA reactivity was selected. The reactivities of 18 selected clones to *E. histolytica* antigen were compared by ELISA. Three of 13 Fabs obtained by shuffling of light chains, CP33-H/L-CP17, CP33-H/L-CP26, and CP33-H/L-LA22, showed relatively high reactivity compared with the reactivity of CP33. The relative optical densities for these Fabs were 1.61. 1.26, and 1.42, respectively, when the optical density of CP33 was defined as 1. Two of the light chains (L-CP17 and L-CP26) were derived from the cyst passer, and one (L-LA22) was derived from the patient with a liver abscess. The reactivities of the five Fabs obtained by shuffling of the heavy chain were similar to or lower than the reactivity of CP33; the relative optical densities were between 0.71 and 0.95.

Gene analysis of recombinant Fabs. Six heavy-chain genes and 14 light-chain genes were sequenced, and the deduced amino acid sequences that they encode were compared. In the heavy chains, H-LA5 derived from the patient with a liver abscess was identical to CP33-H (Fig. 1). H-CP4 also was almost identical to CP33-H except for the FR1 region. In L-LA22, the sequence of complementarity-determining regions (CDRs) was identical to that in CP33-L (Fig. 2). The difference between L-CP17 and CP33-L in the CDRs was only one amino acid residue in CDR3. In L-CP26, three residues in CDR1 and four residues in CDR3 differed from residues in CP33-L. The sequence homology of these clones with germ lines was analyzed by using IgBLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/igblast/) and V-QUEST at the international ImMunoGeneTics database (http://imgt.cines.fr:8104/textes/vquest/). As shown in Table 1, sequence analysis of the heavy-chain genes revealed that all of the most similar germ line of V-segments belonged to the VH3 family (VH3-21, VH3-30, VH3-48, or VH3-53). However, the level of amino acid homology with the germ line was low, 76% in CP33-H and H-LA5. The most similar germ line for the D-segment was D2-2 or D6-6, except for H-CP1, which was not identified, and the most similar germ line for the J-segment was either JH4b or JH6b. In contrast, all the light chains belonged to the Vκ1 family, in which the most similar

```
                FR1                 CDR1            FR2             CDR2
CP33-L  DIVMTQTPSSLSASVGDRVTITC RASQSISSYLN WYQQRPGQAPKLLIY AASSLQS
L-CP9   ..EL..S..T..... ....G...W.A ....K..K..... .......
L-CP13  ..Q...S...V.....T..... ....T..... ....K..K...V... .......
L-CP17  A.Q...S..... ..... ....K..K..... .......
L-CP26  ..QL..S...V..... ....G...W.A ....K..K..... .......
L-LA2   ..Q...S...V..... ....G..RW.V ....K..K..... G..T...
L-LA3   ...L..S...V..... ....G...W.A ....K..K..... .......
L-LA8   ......S...V.....A... ....G..NW.A ....K..K..N...F ...T..T
L-LA10  ......S..... ..... ....K..K..... .......
L-LA11  A.QL..S..... .....V.TF.S ....K..K..... .......
L-LA12  E..L..S..YV..... ....G.GRW.G ...HK.....N.... ...T..G
L-LA14  A.QL..S...A.....E..... ....G.NNW.G ....K..K..... .......
L-LA18  A.QL..S..... .....VTT... ....K..K..... S......
L-LA22  ..E...S..... ..... ....K..K..... .......

                FR3                 CDR3        FR4
CP33-L  GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQSYSTPRTF GQGTRLDIKR
L-CP9   ..... ..AN.F.L.. .G..KVE...
L-CP13  ..... ..... ....KVE..G
L-CP17  ..... .....H.. ....K.E...
L-CP26  ..... ..ANNF.... ....KVE...
L-LA2   .....N.....H. ..TD.L.L.. .G..KVE.R.
L-LA3   .....S.S... ..TN.F.I.. ......E...
L-LA8   ..... ..AH.I.L.. .G..KVE...
L-LA10  .....A... .....Y.. ....K.E...
L-LA11  .....A..... .....I.Y.. ....K.E...
L-LA12  .....S.A... ..AN.F.... ....KVE...
L-LA14  ..... ...N.F.... ....KVE...
L-LA18  .....S..... .....I.. ......E...
L-LA22  ..... ..... ....KVE...
```

FIG. 2. Deduced amino acid sequences for genes coding for light-chain variable regions of human anti-*E. histolytica* Fab fragments. FR, framework regions. The dashes and dots indicate deletions and identical residues, respectively.

Vκ germ line was 02/012 or L5, with relatively high homology (Table 2). For the Jκ segments, Jκ1, Jκ2, and Jκ5 were combined with 02/012, and Jκ1, Jκ2, and Jκ4 were combined with L5.

Specificity of recombinant Fabs. CP33 and the three Fabs which showed high reactivity in ELISA (CP33-H/L-CP17, CP33-H/L-CP26, and CP33-H/L-LA22) were purified by His affinity column chromatography. SDS-PAGE demonstrated

TABLE 1. Comparison of gene usage and structural homologies for heavy-chain variable regions of anti-*E. histolytica* Fab fragments

Clone	V-segment				D-segment	J-segment
	VH family	Closest germ line	% Homology with germline: DNA	% Homology with germline: Protein		
CP33-H	VH3	VH3-30	85	76	D2-2	JH6b
H-CP1	VH3	VH3-21	88	80	ND[a]	JH6b
H-CP4	VH3	VH3-30	85	78	D2-2	JH6b
H-CP5	VH3	VH3-53	92	87	D6-6	JH4b
H-CP6	VH3	VH3-48	95	88	D2-2	JH4b
H-LA5	VH3	VH3-30	85	76	D2-2	JH6b

[a] ND, not determined.

TABLE 2. Comparison of gene usage and structural homologies for light-chain variable regions of anti-*E. histolytica* Fab fragments

Clone	V-segment				J-segment
	VL family	Closest germ line	% Homology with germline: DNA	% Homology with germline: Protein	
CP33-L	Vκ1	02/012	98	95	Jκ5
L-CP9	Vκ1	L5	97	95	Jκ4
L-CP13	Vκ1	02/012	97	95	Jκ1
L-CP17	Vκ1	02/012	99	100	Jκ2
L-CP26	Vκ1	L5	97	93	Jκ1
L-LA2	Vκ1	L5	94	90	Jκ4
L-LA3	Vκ1	L5	98	94	Jκ5
L-LA8	Vκ1	L5	95	90	Jκ4
L-LA10	Vκ1	02/012	99	97	Jκ2
L-LA11	Vκ1	02/012	96	92	Jκ2
L-LA12	Vκ1	L5	93	85	Jκ1
L-LA14	Vκ1	L5	95	92	Jκ1
L-LA18	Vκ1	02/012	98	93	Jκ5
L-LA22	Vκ1	02/012	99	98	Jκ1

that the molar ratio of two bands with apparent molecular masses of 25 and 24 kDa was 1:1, suggesting the heterodimeric structure of Fab. These four Fabs reacted with all 10 strains of *E. histolytica* trophozoites, but not with *E. dispar* trophozoites, in IFA when fixed cells were used (data not shown). To identify the *E. histolytica* antigen recognized by these Fabs, a Western immunoblot analysis was performed. Under nonreducing conditions, four Fabs were reactive only with a 260-kDa antigen (Fig. 3). To identify the antigen, a dot immunoblot analysis was carried out. Four Fabs reacted with the affinity-purified 260-kDa Gal/GalNAc lectin and also with the cysteine-rich domain of the heavy subunit of the lectin (Fig. 4).

Affinity of recombinant Fabs. The affinity of the Fabs to the lectin was measured by surface plasmon resonance. The association constants for the four Fabs with the 260-kDa lectin ranged from 1.06×10^8 to 2.85×10^8 M^{-1} (Table 3). Although the affinity values for rLecA were low, they ranged from 6.43×10^7 to 1.29×10^8 M^{-1}. CP33-H/L-CP26 and CP33-H/L-LA22 showed higher affinity than CP33 and CP33-H/L-CP17 showed.

Neutralizing activity of recombinant Fabs. To evaluate the function of the four Fabs, their effects on amebic adherence to CHO cells were examined. In a normal human Fab-treated control, the level of adherence of trophozoites to CHO cells was 53.8%. After pretreatment of trophozoites with 100 μg of the antilectin Fabs, the level of adherence significantly decreased by 57 to 65% ($P < 0.001$). The effect of the Fabs on erythrophagocytosis by *E. histolytica* was also evaluated. When amebae were pretreated with 100 μg of the Fabs, the number of trophozoites ingesting erythrocytes and the number of erythrocytes ingested were significantly decreased (Table 4). No significant differences in inhibitory effects on adherence and erythrophagocytosis were seen among the four Fabs.

DISCUSSION

Our findings demonstrate that neutralizing human antibodies to amebic adherence and erythrophagocytosis can be prepared from an immunoglobulin gene library derived from an asymptomatic cyst passer infected with *E. histolytica* but not

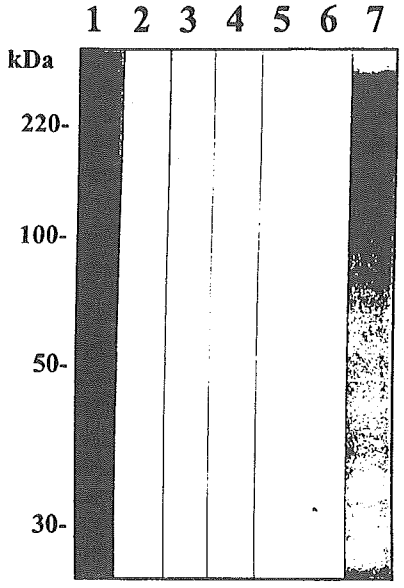

FIG. 3. Western immunoblot analysis of reactivities of human monoclonal antibody Fab fragments with trophozoites of *E. histolytica* HM-1:IMSS. Cell lysates were subjected to SDS-PAGE in a 7.5% polyacrylamide gel under nonreducing conditions and transferred to polyvinylidene difluoride membranes. The protein bands in lane 1 were stained with Coomassie brilliant blue. Lane 2, CP33; lane 3, CP33-H/L-CP17; lane 4, CP33-H/L-LA22; lane 5, CP33-H/L-CP26; lane 6, *E.coli* lysates (vector control); lane 7, plasma from a patient with an amebic liver abscess. The preparations in lanes 2 to 7 were treated with HRP-conjugated sheep antibody to human IgG $F(ab')_2$. The numbers on the left indicate the molecular masses of size markers.

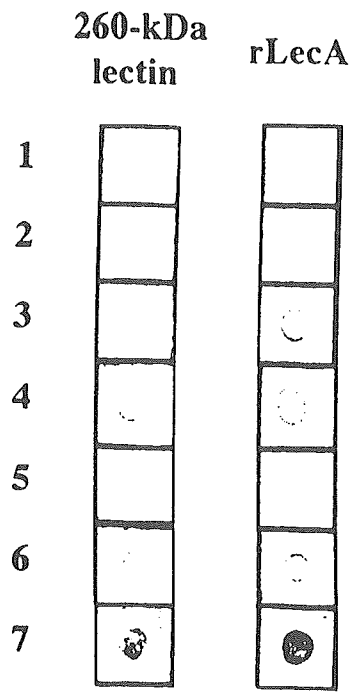

FIG. 4. Dot immunoblot analysis of reactivities of human monoclonal antibody Fab fragments with an affinity-purified 260-kDa lectin and with rLecA. One microgram of the 260-kDa lectin and 1 μg of rLecA were blotted onto a nitrocellulose membrane. The spots were treated as follows: line 1, CP33; line 2, CP33-H/L-CP17; line 3, CP33-H/L-LA22; line 4, CP33-H/L-CP26; line 5, control human Fab; line 6, IgG purified from plasma of a patient with an amebic liver abscess; line 7, anti-260-kDa lectin rabbit antibody. Then the preparations were treated with HRP-conjugated sheep antibody to human IgG $F(ab')_2$ (lines 1 to 6) or HRP-conjugated goat antibody to rabbit IgG (line 7).

with *E. dispar*. The epitope recognized by CP33 was located in a cysteine-rich domain of the heavy subunit of the Gal/GalNAc lectin. To date, seven different mouse monoclonal antibodies specific for nonoverlapping epitopes on the cysteine-rich domain of the heavy subunit of lectin have been identified (28, 35). Three of the seven murine antibodies inhibited amebic adherence to target cells, but two enhanced adherence by causing a marked increase in the galactose-binding activity of the lectin. Since the human antibodies prepared in this study had an inhibitory effect on amebic adherence to CHO cells, these antibodies must recognize an adherence-inhibiting epitope in the cysteine-rich domain (28). This conclusion is also supported by the fact that CP33 did not react with *E. dispar*, as previously it has been shown that the adherence-inhibiting epitopes are *E. histolytica* specific (28, 33). Demonstration of the ability of the Fabs to inhibit erythrophagocytosis indicates involvement of the Gal/GalNAc lectin in this process for the first time. Erythrophagocytosis is of interest as it is a characteristic property that distinguishes *E. histolytica* from the nonpathogenic parasite *E. dispar*.

In a previous study, the immunogobulin gene library derived from a patient with an amebic liver abscess was screened by the methods used in the present study. The positive rate of the first screening was 0.054% (27 of 5×10^4 of clones were positive), which is 5.7-fold higher than the rate (0.0095%) observed in this study (9). However, there was only one positive clone in the second screening by IFA with intact trophozoites. This suggests that the proportion of antibodies recognizing the trophozoite surface in the symptomatic patient with a liver abscess was smaller than the proportion in the asymptomatic cyst passer, even though the anti-*E. histolytica* antibody titer was higher in the symptomatic patient.

In the present study, when the heavy or light chain of CP33 was recombined with genes from the two libraries and rescreened, the positive rates were higher in the LA library. This may have been due to the symptomatic patient with the amebic liver abscess having a high antibody titer. However, the relative values were decreased to 2- or 4-fold from 5.7-fold. We concluded from these results that asymptomatic cyst passers have a high ratio of antibodies recognizing the adherence-inhibiting epitope of the heavy-subunit lectin compared with the ratio in

TABLE 3. Association and dissociation constants for the binding of recombinant human Fabs to the 260-kDa lectin and rLecA, as measured by surface plasmon resonance[a]

Fab	260-kDa lectin		rLecA	
	K_a (1/M)	K_d (M)	K_a (1/M)	K_d (M)
CP33	1.06×10^8	9.40×10^{-9}	7.19×10^7	1.39×10^{-8}
CP33-H/L-CP17	1.19×10^8	8.42×10^{-9}	6.43×10^7	1.56×10^{-8}
CP33-H/L-CP26	2.09×10^8	4.78×10^{-9}	1.19×10^8	8.40×10^{-9}
CP33-H/L-LA22	2.85×10^8	3.51×10^{-9}	1.29×10^8	7.73×10^{-9}

[a] K_a, association constant; K_d, dissociation constant.

TABLE 4. Effect of recombinant Fabs on erythrophagocytosis by *E. histolytica*[a]

Fab	% Of amebae with red blood cells (*P* vs control)	No. of red blood cells per ameba (*P* vs control)
Control	68.5 ± 5.9	2.70 ± 0.53
CP33	35.0 ± 6.1 (<0.005)	0.64 ± 0.17 (<0.005)
CP33-H/L-CP17	36.9 ± 3.5 (<0.002)	0.70 ± 0.07 (<0.005)
CP33-H/L-CP26	46.8 ± 6.2 (<0.02)	1.07 ± 0.25 (<0.01)
CP33-H/L-LA22	38.5 ± 7.8 (<0.01)	0.76 ± 0.15 (<0.005)

[a] Trophozoites (10^4 cells) were treated with 100 μg of Fab before incubation with human erythrocytes (10^6 cells). The results are means ± standard deviations for data from three experiments.

symptomatic patients. These adherence-inhibiting antibodies may help prevent the invasion of trophozoites into tissues in cyst passers, although no information was obtained in this study concerning antibodies to other adherence-inhibiting epitopes.

When the heavy chain of CP33 was combined with the light chains from the libraries, the positive rates for screening by colony blotting were 10- to 20-fold higher than the positive rates for screening of combinations of light chains of CP33 with heavy chains. This suggests that the heavy chain is more important for the binding of antibodies to the lectin. Indeed, the fact that the V-segment gene sequence of CP33-H contains many somatic mutations and the fact that no gene homologous with CP33-H has been reported are in accord with the observation that CP33 is reactive specifically with *E. histolytica*. Heavy-chain dominance in determining antigen binding has been demonstrated for antibodies to gp120 and to the reverse transcriptase of human immunodeficiency virus type 1 (HIV-1) (6, 26). In contrast, it has been reported that DNA binding activity is determined by the light chains in human anti-double-stranded DNA IgG Fab clones (41).

The present study revealed that all the most similar V-segment germ lines of the cloned heavy chain belonged to the VH3 family. The VH3 gene family, with 22 functional genes, is the largest of the seven families (VH1 to VH7) and comprises about one-half of the expressed VH repertoire in adult peripheral B cells (13, 29). However, biases in gene family usage of the heavy-chain variable region have been reported in a number of diseases. For example, restricted VH3 germ line gene usage was observed in intravenous immunoglobulin-bound Fabs from a patient with thrombocytopenia that had progressed to systemic lupus erythematosus (31). It is known that VH3 antibodies are also important for defense against a variety of bacteria (1, 39, 40) and viruses (2, 17, 20). Although VH3 antibodies bind to HIV-1 gp120 in HIV-infected late-stage patients, VH3 gene family expression is reduced compared with the expression in healthy donors, but the other two main VH gene families, VH1 and VH4, show no significant variation in expression (14). When the gene usage of another neutralizing anti-*E. histolytica* lectin Fab, LA-01, which had previously been prepared from an LA library (9), was examined, it was found that the most similar germ lines of LA-01 were VH3-30, D1-26, and JH6c for the heavy chain and Vκ02/012 and Jκ5 for the light chain. This observation also supports the preferential usage of the VH3 gene family for the adherence-inhibiting epitope of the lectin. To our knowledge, this report is the first report demonstrating that VH3 antibodies are important in defense against parasitic infections.

In contrast to heavy-chain gene usage, the light-chain gene repertoire of human antibodies to HIV does not exhibit a family bias (15). In the present study, all 14 light chains from both libraries belonged to the Vκ1 family, in which the closest Vκ germ lines were 02/012 and L5, in spite of the selection of clones showing different patterns after restriction endonuclease digestion. This finding demonstrates that a limited repertoire of light-chain genes is required to create a functional binding site with the heavy chain of CP33. This is in accord with previous reports that some heavy chains prefer to pair with similar light-chain variable regions to form high-affinity binders (3, 21, 30).

In the present study, we found that there was only one amino acid residue that was different in CDR3 when CP33-L and L-CP17 were compared. One of the advantages of recombinant antibody technology is possible modification of the original antibody gene. By introduction of synthetic genetic variability in CDR3, which is an important region in antigen binding, it may be possible to increase the affinity and/or neutralizing activity of the antibody.

Whereas the advantage of a phage display system for the preparation of human antibodies has recently been demonstrated (5, 19, 36), the present study shows that screening by colony blotting and chain shuffling of cloned genes may be a useful way to find genes of immunoglobulins with high affinity to pathogens. Total analysis of antibody genes for the amebic lectin, including other adherence-inhibiting epitopes on the heavy and intermediate subunits of the lectin, should be helpful not only for understanding the mechanism of protective immunity but also for development of immunoprophylaxis against invasive amebiasis.

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Entamoeba invadens: inhibition of excystation and metacystic development by aphidicolin

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Abstract

The effect of aphidicolin, a specific inhibitor of the replicative DNA polymerases, on the excystation and metacystic development of *Entamoeba invadens* was examined. The protein profile of metacystic amoebae and their immunogenicity in the presence and absence of aphidicolin were also examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting. Excystation, which was assessed by counting the number of metacystic amoebae after the induction of excystation, was inhibited by aphidicolin in a concentration-dependent manner during incubation compared to the controls. Metacystic development, when determined by the number of nuclei in amoeba, was also inhibited by aphidicolin, because the percentage of 4-nucleate amoebae in cultures with aphidicolin during incubation was higher than that in cultures without the drug. The addition of aphidicolin to cultures at day 1 of incubation reduced the number of metacystic amoebae thereafter compared to cultures without the drug. The inhibitory effect of aphidicolin on excystation and metacystic development was reversed by removal of the drug. Pretreatment of cysts with aphidicolin before transfer to a growth medium containing the drug had no further effect on the excystation and metacystic development. Cellular proteins of metacystic amoebae with 4 nuclei, which were predominant even at day 3 in the cultures with aphidicolin, reacted strongly with rabbit anticyst serum absorbed with trophozoite proteins. In contrast, those of metacystic amoebae with 1 nucleus, which were predominant at day 3 in cultures without aphidicolin, no longer reacted with the absorbed anticyst serum, suggesting change in the expression of proteins during metacystic development.

Index Descriptors and Abbreviations: Entamoeba invadens; protozoa; aphidicolin; DNA polymerase; excystation; metacystic development; immunogenicity; DNA, deoxyribonucleic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide.

1. Introduction

Aphidicolin is a mycotoxin produced by fungi such as *Cephalosporium aphidicola* and *Nigrospora oryzae*. This tetracyclic diterpenoid is known as a specific inhibitor of nuclear replicative DNA polymerases in eukaryotic cells (reviewed by Spadari et al., 1982; Wang, 1991). Aphidicolin blocks eukaryotic cells in the S phase by inhibiting the replicative DNA polymerase and allows G2, M, and G1 cells to accumulate specifically at the G1/S border; it does not reduce cell viability and its action is reversible (Pedrali-Noy et al., 1980).

It was previously demonstrated that aphidicolin inhibits *Entamoeba histolytica* growth and DNA synthesis and induces synchronous growth of the parasite in the recovery phase after the removal of aphidicolin (Makioka et al., 1998). The inhibitory effects of aphidicolin were also demonstrated on the growth and encystation of *Entamoeba invadens* and the reversibility of its action (Kumagai et al., 1998). Thus aphidicolin is considered a useful tool for studies on cellular processes relating to DNA synthesis.

Excystation and metacystic development are necessary for *Entamoeba* infection and their processes were described for *E. histolytica* previously (Cleveland and Sanders, 1930; Dobell, 1928). However, knowledge of the excystation as well as the encystation is limited since no axenic encystation medium is available for this

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parasite (López-Romero and Villagómez-Castro, 1993). Studies have been conducted on the axenic in vitro encystation of *E. invadens*, a reptilian parasite, because of its close similarity with *E. histolytica* in morphology and life cycle (McConnachie, 1969). Since the excystation and metacystic development of *E. histolytica* (Cleveland and Sanders, 1930; Dobell, 1928) and those of *E. invadens* (Geiman and Ratcliffe, 1936) look similar, in vitro excystation of *E. invadens* may also become a useful model for excystation of the human parasite. Excystation is the process whereby the whole organism escapes from the cyst through a minute perforation in the cyst wall. Metacystic development is the process whereby a hatched metacystic amoeba with 4 nuclei divides to produce 8 amoebulae, which grow to become trophozoites (Cleveland and Sanders, 1930; Dobell, 1928; Geiman and Ratcliffe, 1936). The transfer of *E. invadens* cysts in an encystation medium to a growth medium could induce in vitro excystation (Garcia-Zapien et al., 1995; McConnachie, 1955; Rengpien and Bailey, 1975). No study has been conducted to follow the changes in the protein profile and their immunogenicity during excystation and metacystic development. Rabbit antisera were previously prepared against trophozoites and cysts, and the appearance of cyst-specific proteins was demonstrated in encysting *E. invadens* and their immunogenicity (Makioka et al., 2000). The present study examined the effect of aphidicolin on the excystation and metacystic development of *E. invadens* and also followed the changes occurring in the protein profile during these processes in the presence of aphidicolin by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting using these antisera. Here, it is reported that aphidicolin inhibits the excystation and metacystic development of *E. invadens* and also affects expression of proteins of metacystic amoebae that are recognized by cyst and trophozoite-specific antisera during development.

2. Materials and methods

Trophozoites of the IP-1 strain of *E. invadens* were cultured in axenic growth medium BI-S-33 (Diamond et al., 1978) at 26 °C. To obtain cysts, trophozoites (5×10^5 cells/ml) were transferred to an encystation medium called 47% LG (LG is BI without glucose; Sanchez et al., 1994). After 3 days of incubation, the cells were harvested and treated with 0.05% Sarkosyl (Sigma Chemical, St. Louis, MO) to destroy the trophozoites (Sanchez et al., 1994). The remaining cysts were washed with phosphate-buffered saline (PBS), counted, and suspended in a growth medium. Viability of the cysts was determined by trypan blue dye exclusion. For experiments on the excystation and metacystic development of *E. invadens*, duplicate cultures of 5×10^5 cysts/ml were incubated with 0.1–10 μg/ml aphidicolin for 3 days. Metacystic amoebae were counted in a hemocytometer at 5 h (0 and 10 μg/ml only), days 1, 2, and 3 and their viability determined by trypan blue dye exclusion. Viable metacystic amoebae and cysts were clearly distinguished as light yellow and light blue in color, respectively. The former was also identified by positive motility. Aphidicolin, purchased from Sigma, was initially dissolved in dimethyl sulfoxide (DMSO). The control cultures received the same volume of DMSO.

Metacystic development was determined by the number of nuclei per amoeba. Cells were harvested at days 1 and 3 in cultures with or without 10 μg/ml aphidicolin and stained with modified Kohn (Kumagai et al., 2001). The number of nuclei per amoeba was determined by double-counting at least 100 amoebae.

For experiments on the effect of aphidicolin on excystation and metacystic development after the induction of excystation, duplicate cultures (5×10^5 cysts/ml) were incubated for 1 day, then aphidicolin was added to one culture at a concentration of 10 μg/ml. The cultures were incubated for an additional 2 days, and the metacystic amoebae were counted.

For experiments on the reversibility of the effect of aphidicolin, duplicate cultures containing 10 μg/ml aphidicolin were incubated for 1 day. Cells were then centrifuged at 400*g* for 5 min after chilling on ice and the spent medium removed. Cells were washed twice with a growth medium and then suspended in a fresh growth medium. In control cultures, cells were similarly treated without replacement of the medium. The cultures were incubated for an additional 2 days, and the metacystic amoebae were counted.

To examine the effect of pretreatment with aphidicolin on excystation and metacystic development, cysts were first incubated for 30 min in an encystation medium with or without 10 μg/ml aphidicolin, then transferred to a growth medium with or without the same concentration of drug, and the metacystic amoebae were counted daily.

To examine the change in the protein profile and immunogenicity of metacystic amoebae during development, cellular proteins of metacystic amoebae were prepared as follows: cells were harvested at days 1 and 3 of incubation in cultures with and without 10 μg/ml aphidicolin, washed by centrifugation in PBS, and treated at room temperature for 10 min with 0.05% Sarkosyl containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino) butane (E-64), 1 μg/ml *N*-tosyl-L-lysyl chloromethyl ketone (TLCK), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM benzamidine hydrochloride to disrupt the metacystic amoebae. All these proteinase inhibitors were purchased from Sigma. After centrifugation, the lysates of metacystic amoebae were obtained as supernatants. The lysates of trophozoites were similarly prepared.

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the lysates of metacystic amoebae and trophozoites were mixed with the sample buffer (Laemmli, 1970), cysts were dissolved in the sample buffer and subjected to electrophoresis: 10^5 equivalent of each per lane. Electrophoresis was performed using a Tris–glycine buffer (pH 8.3) containing 0.1% SDS (Laemmli, 1970) on 12.5% polyacrylamide slab gels. The gels were stained with Coomassie blue R-250.

For immunoblotting, the nitrocellulose filters were exposed for 1 h to a 1:100 dilution of rabbit antitrophozoite serum, anticyst serum or the anticyst serum absorbed with trophozoite proteins, in PBS containing 0.05% Tween 20 (PBS/Tween). The preparation of these antisera was described previously (Makioka et al., 2000). The filters were washed with PBS/Tween, incubated for 1 h with peroxidase-conjugated goat anti-rabbit immunoglobulins (ICN Pharmaceuticals) diluted in 1:1000 in PBS/Tween, washed again, and developed with PBS containing 4-chloro-1-naphthol and hydrogen peroxide.

All the experiments of the present study were performed at least three times and similar results were obtained. Therefore, the data presented in the results are representative.

3. Results

The effect of aphidicolin on the number of metacystic amoebae of *E. invadens* after the transfer of cysts to a growth medium was examined. At 5 h of incubation, the number of metacystic amoebae in cultures without aphidicolin was 1.84 ± 0.19 ($\times 10^4$/ml), whereas it was 0.96 ± 0.06 ($\times 10^4$/ml) ($P < 0.05$) in cultures containing 10 μg/ml of the drug. At day 1, little decrease in the number of metacystic amoebae occurred in cultures with 0.1 μg/ml aphidicolin, whereas it significantly decreased in cultures with more than 0.5 μg/ml drug compared to the controls (Fig. 1). Amoebae further increased in number from day 1 to day 3 of incubation in cultures without the drug. In contrast, metacystic amoebae remained unchanged in number from day 1 to day 3 in cultures containing 5 and 10 μg/ml.

The effect of aphidicolin on cyst viability is shown in Fig. 2. The number of viable cysts in cultures containing aphidicolin during incubation was comparable to or greater than that of controls.

The effect of aphidicolin on metacystic development was examined by counting the number of nuclei per cell. As shown in Fig. 3, 53% of metacystic amoebae were 4-nucleate at 5 h of incubation in both cultures with and without aphidicolin. The percentage of 4-nucleate amoebae in the control cultures then decreased to 17 and 2% at days 1 and 3, respectively, following increased percentages of 1- to 3-nucleate amoebae. In contrast, the

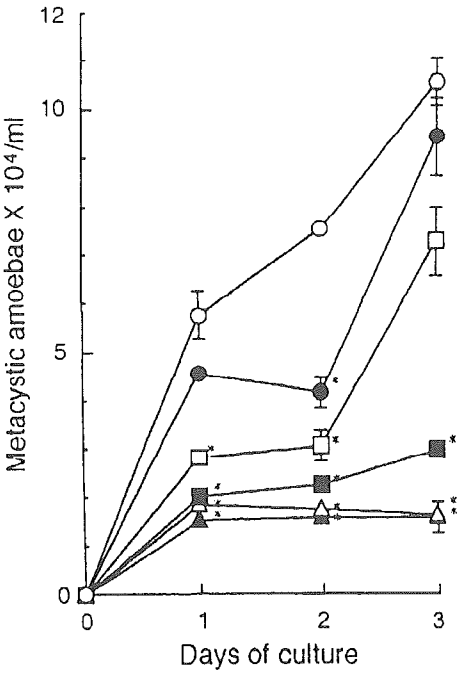

Fig. 1. Effect of aphidicolin on the number of metacystic amoebae of *E. invadens*. Cysts were transferred to a growth medium containing various concentrations of aphidicolin. The mean number ± SE of metacystic amoebae for duplicate cultures is plotted (*$P < 0.05$). Concentrations of 0, 0.1, 0.5, 1, 5, and 10 μg/ml are shown by open circles, solid circles, open squares, solid squares, open triangles, and solid triangles, respectively.

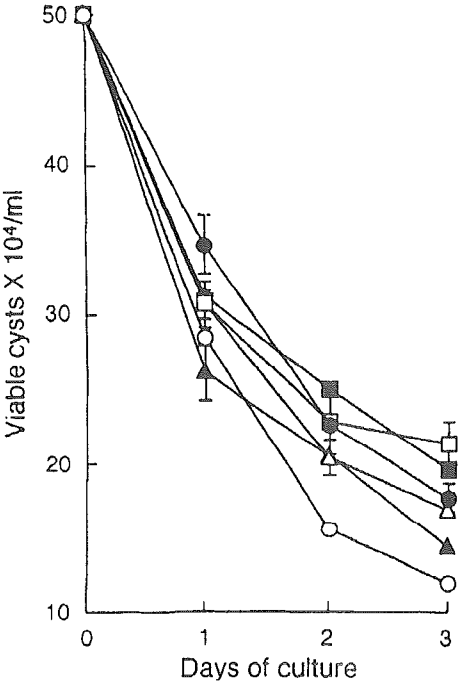

Fig. 2. Effect of aphidicolin on cyst viability in the growth medium. Experimental conditions were the same as those for Fig. 1. The mean number ± SE of viable cysts for duplicate cultures is plotted. Concentrations of 0, 0.1, 0.5, 1, 5, and 10 μg/ml are shown by open circles, solid circles, open squares, solid squares, open triangles, and solid triangles, respectively.

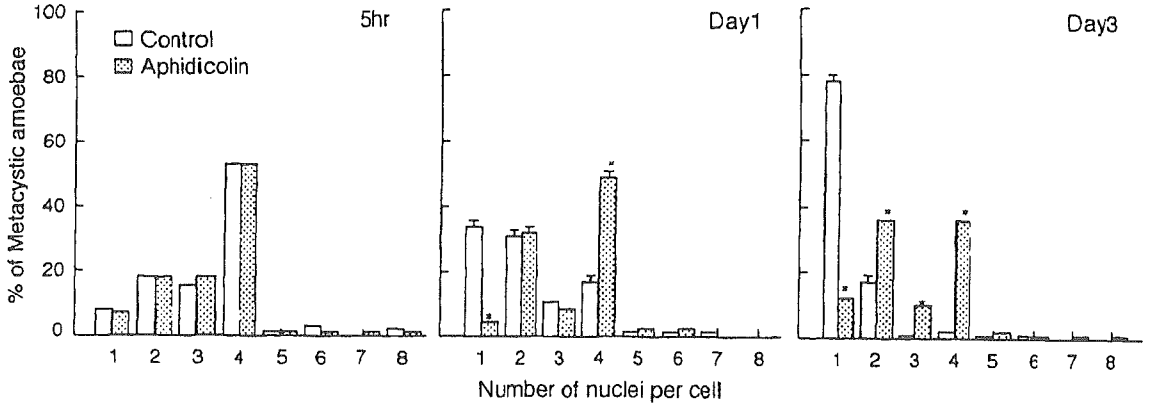

Fig. 3. Effect of aphidicolin on the metacystic development of *E. invadens*. Cysts were transferred to a growth medium with or without 10 μg/ml aphidicolin. The numbers of nuclei per metacystic amoeba stained with modified Kohn at 5 h and days 1 and 3 of incubation were counted and the percentage of amoebae was determined (*$P < 0.05$).

percentage of 4-nucleate amoebae in cultures with aphidicolin was still 49 and 36% at days 1 and 3, respectively, suggesting the inhibition of metacystic development by aphidicolin.

To examine the effect of aphidicolin on excystation and metacystic development after induction, aphidicolin (10 μg/ml) was added to cultures at day 1 of incubation and metacystic amoebae counted 1, 2, and 3 days later. As shown in Fig. 4, metacystic amoebae markedly decreased in number 1 and 2 days after the addition of aphidicolin compared with those in cultures without the drug. The reason for this decrease remains unclear. The percentages of 4- and 1- to 3-nucleate amoebae in cultures 1 day after the addition of aphidicolin were 8 and 88%, respectively, which were almost the same as those of 9 and 89%, respectively, in the control cultures.

To determine whether the inhibitory effect of aphidicolin on excystation and metacystic development was reversible, spent medium containing 10 μg/ml aphidicolin for 1 day of incubation was replaced with a drug-free growth medium. After removal of the drug, the number of metacystic amoebae increased to 91% of the control (Fig. 5). The recovery of the number of amoebae was associated with the increase in percentage of 1- to 3-nucleate amoebae (data not shown).

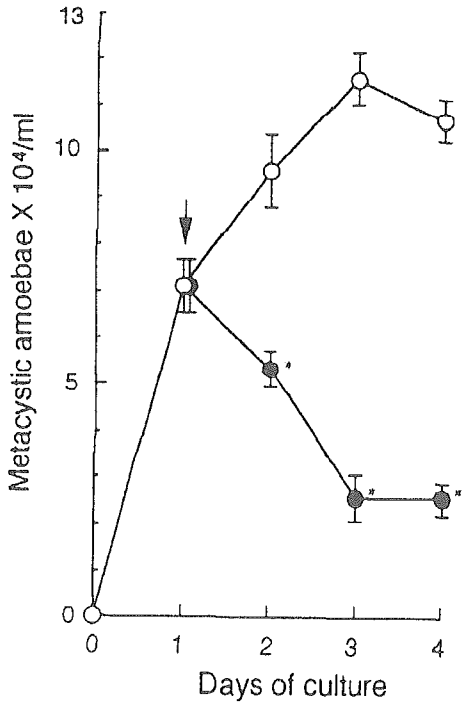

Fig. 4. Effect of aphidicolin on the excystation and metacystic development of *E. invadens* after the induction of excystation. At day 1 of incubation, aphidicolin (10 μg/ml) was added to cultures and metacystic amoebae counted for another 3 days (*$P < 0.05$).

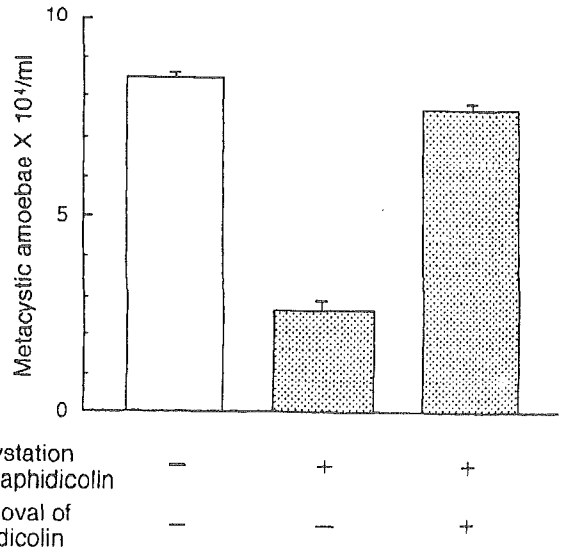

Fig. 5. Effect of the removal of aphidicolin on the excystation and metacystic development of *E. invadens*. After exposure of cysts to 10 μg/ml aphidicolin in a growth medium for 1 day, the drug was removed by replacement of the medium with a drug-free growth medium and the cultures were further incubated for 3 days. The mean number + SE of metacystic amoebae for duplicate cultures is plotted.

To examine the effect of pretreatment with aphidicolin on excystation and metacystic development, cysts were exposed to 10 μg/ml aphidicolin in an encystation medium before transfer to a growth medium containing the drug. As shown in Fig. 6, the pretreatment caused no further inhibition of excystation and metacystic development as compared to that without the pretreatment.

To follow the change in the protein profile during excystation and metacystic development in cultures with and without aphidicolin, SDS–PAGE and immunoblotting were conducted. As shown in Fig. 7A, the protein profiles of metacystic amoebae were almost the same as those of cysts and trophozoites, but the protein bands of metacystic amoebae at day 3 in control cultures were weakly stained compared to those of other forms. As shown in Fig. 7B, not only trophozoite proteins but also a number of proteins of metacystic amoebae and cyst proteins were immunostained with rabbit antitrophozoite serum. Also, the proteins of metacystic amoebae at day 1 in cultures with aphidicolin were the most weakly immunostained.

When nitrocellulose filters were immunostained with rabbit anticyst serum, many metacystic amoeba proteins as well as cyst and trophozoite proteins were also immunostained (Fig. 8B). Also, proteins of metacystic amoebae at day 3 in the control cultures and trophozoites were weakly immunostained compared to the others. When the anticyst serum absorbed with trophozoite proteins was used, neither metacystic amoeba proteins at day 3 in control cultures nor trophozoite proteins were immunostained, whereas the 88- and 66-kDa cyst proteins of metacystic amoebae at day 1 in cultures with and without aphidicolin and those at day 3 in cultures with the drug were strongly immunostained (Fig. 8C).

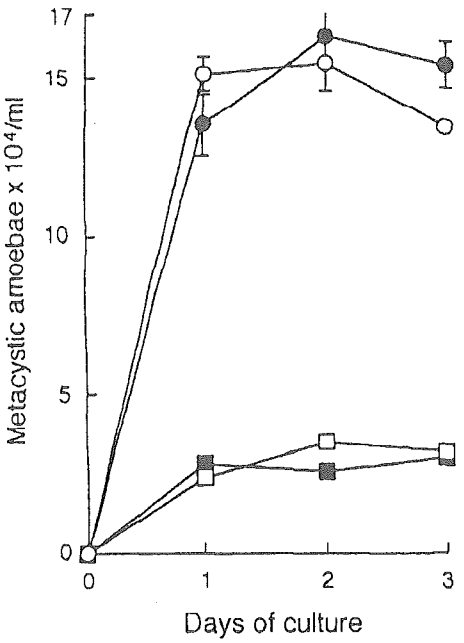

Fig. 6. Effect of pretreatment of aphidicolin on the excystation and metacystic development of *E. invadens*. Cysts were incubated for 30 min in an encystation medium with or without 10 μg/ml aphidicolin before transfer to a growth medium with or without the drug. The mean number ± SE of metacystic amoebae for duplicate cultures is plotted. No pretreatment and no drug in the growth medium (open circles); pretreatment without aphidicolin and growth medium without the drug (solid circles); pretreatment with the drug and growth medium without the drug (open squares); pretreatment without the drug and growth medium with the drug (solid squares); pretreatment with the drug and growth medium with the drug (open triangles).

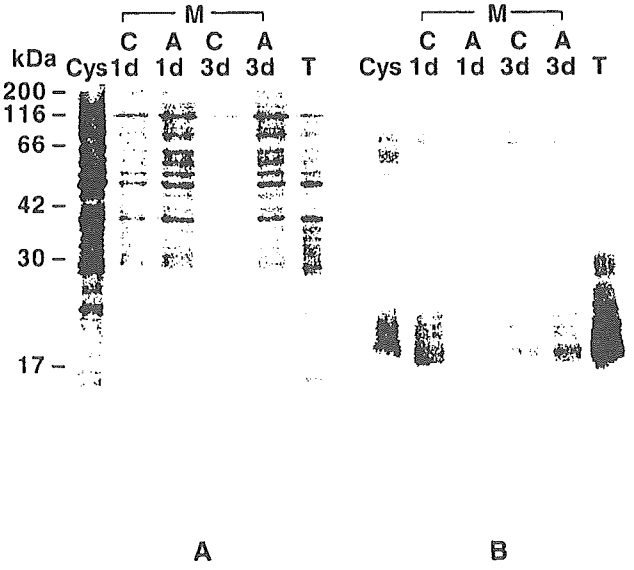

Fig. 7. Immunoblot analysis of cellular proteins that react with rabbit antitrophozoite serum during the metacystic development of *E. invadens*. Metacystic amoebae at days 1 and 3 in cultures with and without aphidicolin as well as cysts and trophozoites were subjected to SDS–PAGE and immunoblotting [Cys, Cysts; M, Metacystic amoebae in cultures with (A) and without (C) aphidicolin; T, Trophozoites]. (A) Protein staining with Coomassie blue; (B) immunostaining with rabbit antitrophozoite serum. The molecular-mass standards are as follows: myosin (200 kDa), β-galactosidase (116 kDa), albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), and myoglobin (17 kDa).

4. Discussion

The results clearly indicate that aphidicolin inhibits the excystation and metacystic development of *E. invadens*. Although it may be difficult to distinguish excystation completely from metacystic development because it does not occur simultaneously (Cleveland and Sanders, 1930; Dobell, 1928), the number of metacystic amoebae at 5 h in cultures containing aphidicolin, at which time the percentage of 4-nucleate amoebae was more than 50% in cultures with and without the drug, was significantly reduced compared to the controls. This suggests that aphidicolin affected the excystation possibly through its inhibition of the replicative DNA

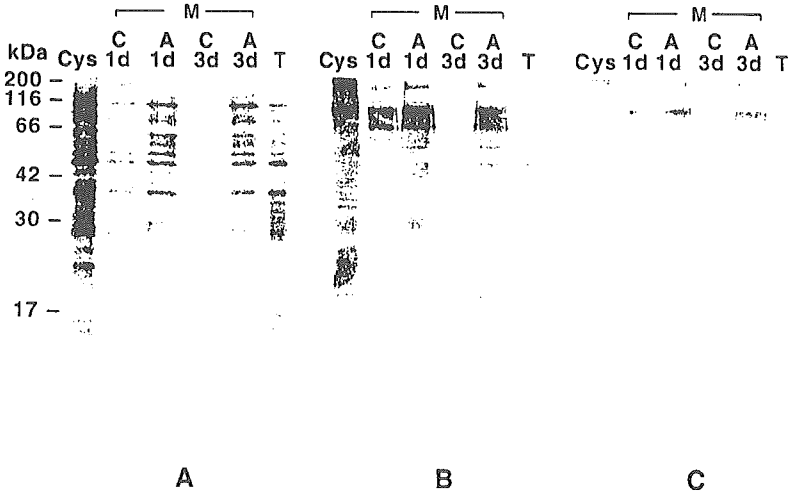

Fig. 8. Immunoblot analysis of cellular proteins that react with rabbit anticyst serum during the metacystic development of *E. invadens*. Conditions for excystation and SDS–PAGE were the same as those described for Fig. 7. (A) Protein staining with Coomassie blue; (B) immunostaining with rabbit anticyst serum; (C) immunostaining with anticyst serum absorbed with trophozoite proteins.

polymerase and thereby, inhibition of DNA synthesis. Since the excystation process does not include nuclear division, it remains unclear how DNA synthesis is related to the process. The results also demonstrated that considerable numbers of the metacystic amoebae with 1–3 nuclei were observed even at 5 h of incubation in cultures with and without 10 μg/ml aphidicolin. This means that those amoebae performed nuclear division even in the presence of aphidicolin. In this regard, although it was originally thought that only mature cysts with 4 nuclei were able to hatch (Cleveland and Sanders, 1930; Dobell, 1928; Geiman and Ratcliffe, 1936), 1- and 2-nucleate cysts, and 4-nucleate cysts with chromatoids of *E. histolytica* were able to hatch (Everritt, 1950; Hegner et al., 1932; Tanabe, 1934; Swartzwelder, 1939). This point may need further study in *E. invadens*.

The hatched 4-nucleate metacystic amoebae grow rapidly and divide to form 8 amoebulae. Since this metacystic development requires DNA synthesis, it is a target of aphidicolin. The inhibition of nuclear division by aphidicolin increased the percentage of 4-nucleate amoebae during incubation. This may become a useful tool for the biochemical characterization of metacystic development. The effect of aphidicolin on excystation and metacystic development of *E. invadens* was reversible, which was the same as that on the growth and encystation as previously demonstrated (Kumagai et al., 1998). The pretreatment of cysts with aphidicolin before transfer to a growth medium containing the drug had no further effect on excystation and metacystic development, suggesting that cysts in the encystation medium are not affected by aphidicolin.

Biochemical characterization of the metacystic amoebae is important to know their biological significance, but it has so far not been conducted. For this, first, an attempt was made to isolate the metacystic amoebae by the Percoll method which was useful for the separation of cysts from trophozoites (Avron et al., 1983), but this was unsuccessful. This suggests that there is a difference in cell density between the metacystic amoebae and trophozoites. Therefore, the lysates of metacystic amoebae were prepared by treatment with sarkosyl, which had no effect on cysts, and the lysates were analyzed by SDS–PAGE and immunoblotting. The protein profile of cysts and trophozoites was almost the same under the conditions of one-dimensional SDS–PAGE and Coomassie blue staining (Makioka et al., 2000). The protein profile of metacystic amoebae was also almost similar to that of cysts and trophozoites, suggesting no occurrence of drastic change in the protein pattern after excystation and during metacystic development. The protein bands of metacystic amoebae at day 3 in the control cultures were weakly stained, suggesting that those amoebae, most of which are 1-nucleate, had less protein content in the amoeba. The antitrophozoite serum immunostained not only trophozoite proteins but also a number of metacystic amoeba and cyst proteins, indicating that these three forms share considerable numbers of proteins. Since the reactivity of metacystic amoeba proteins at day 1 in cultures with aphidicolin was weaker than that in cultures without the drug, those amoebae in which 4-nucleate were predominant, had fewer shared trophozoite proteins due to their early stages of development. The results of the absorbed anticyst serum indicated that cyst-specific proteins were present in the metacystic amoebae with 4 nuclei, but they disappeared in those with 1 nucleus, suggesting change in the expression of proteins during metacystic development.

To the authors' knowledge, the present study is the first analysis of metacystic amoeba proteins by immunoblotting using polyclonal antitrophozoite and anticyst

sera as well as the anticyst serum absorbed with trophozoite proteins. An understanding of excystation and metacystic development will lead to the identification of targets for vaccination and chemotherapy to inhibit *Entamoeba* infection.

In summary, the results show that aphidicolin inhibits both the excystation and metacystic development of *E. invadens* and thereby affected change in the expression of proteins of metacystic amoebae during development.

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Molecular Cloning and Characterization of a Protein Farnesyltransferase from the Enteric Protozoan Parasite *Entamoeba histolytica**

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Genes encoding α- and β-subunits of a putative protein farnesyltransferase (FT) from the enteric protozoan parasite *Entamoeba histolytica* were obtained and their biochemical properties were characterized. Deduced amino acid sequences of the α- and β-subunit of *E. histolytica* FT (*Eh*FT) were 298- and 375-residues long with a molecular mass of 35.6 and 42.6 kDa, and a pI of 5.43 and 5.65, respectively. They showed 24% to 36% identity to and shared common signature domains and repeats with those from other organisms. Recombinant α- and β-subunits, co-expressed in *Escherichia coli*, formed a heterodimer and showed activity to transfer farnesyl using farnesylpyrophosphate as a donor to human H-Ras possessing a C-terminal CVLS, but not a mutant H-Ras possessing CVLL. Among a number of small GTPases that belong to the Ras superfamily from this parasite, we identified *Eh*Ras4, which possesses CVVA at the C terminus, as a sole farnesyl acceptor for *Eh*FT. This is in contrast to mammalian FT, which utilizes a variety of small GTPases that possess a C-terminal C*aa*X motif, where X is serine, methionine, glutamine, cysteine, or alanine. *Eh*FT also showed remarkable resistance against a variety of known inhibitors of mammalian FT. These results suggest that remarkable biochemical differences in binding to substrates and inhibitors exist between amebic and mammalian FTs, which highlights this enzyme as a novel target for the development of new chemotherapeutics against amebiasis.

Ras small GTPases function as a molecular switch of signal transduction in cell proliferation and differentiation (1). Ras small GTPases require a post-translational lipid modification called protein farnesylation in order to become membrane-associated and functional (2). Protein farnesylation, catalyzed by protein farnesyltransferase (FT)[1] (3), which is comprised of two heterologous α- (FTα) and β- (FTβ) subunits, is a major post-translational lipid modification, together with protein geranylgeranylation (3). FT and protein geranylgeranyltransferase type I (GGT-I) catalyze the transfer of the farnesyl and geranylgeranyl group from farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate, respectively, to the cysteine residue of a C-terminal C*aa*X of small GTPases including Ras, Rac, and Rho, where C is cysteine, *a* is usually an aliphatic amino acid, and *X* is any amino acid. Marked differences in substrate specificity have been shown between FT and GGT-I, *i.e.* FT mainly utilizes, as substrates, small GTPases possessing the terminal C*aa*X motif, when *X* is serine, methionine, glutamine, cysteine, or alanine (4), whereas GGT-I prefers proteins with the C-terminal C*aa*L or C*aa*F motif (4). Among well characterized Ras proteins that terminate with a C*aa*X motif, human H-Ras-CIMF, N-Ras-CVVM, K-RasA-CIIM, and Rap2-CNIQ are known to be farnesylated by FT, while Rap1A-CLLL, as well as Rho family proteins are geranylgeranylated by GGT-I. It has also been shown that K-RasB-CVIM can be either farnesylated by FT or geranylgeranylated by GGT-I (5). Since constitutively active mutations of Ras proteins have been shown to induce carcinogenesis (6–8), which is suppressed by the inhibition of farnesylation, FT has attracted attention as a target of cancer chemotherapy (9). In addition, several compounds targeting FT have proven promising against African sleeping sickness caused by *Trypanosoma brucei* and Malaria caused by *Plasmodia* species (10).

Entamoeba histolytica is an intestinal protozoan parasite, which causes amebic dysentery, colitis, and liver abscess in humans, and is responsible for an estimated 50 million cases of amebiasis and 40–100 thousand deaths annually (11). A number of small GTPases have been studied including Ras/Rap (12, 13), Rho/Rac (14–18), and Rab (19–21). The completion of the *E. histolytica* genome data base will help us to comprehensively understand the presence and complexity of these small

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB083372 (FTα of *E. histolytica*), AB083373 (FTβ of *E. histolytica*), AB112425 (*Eh*Ras3), and AB112426 (*Eh*Ras4).

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[1] The abbreviations used are: FT, protein farnesyltransferase; GGT-I, protein geranylgeranyltrasferase type I; FTα, α-subunit of farnesyltransferase; FTβ, β-subunit of protein farnesyltransferase; *Eh*FT, farnesyltransferase of *E. histolytica*; FPP, farnesyl pyrophosphate; NTA, nitrilotriacetic acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate, NJ, neighbor-joining.

GTPases in the ameba. The molecular and cellular functions of some of these small GTPases have begun to be unveiled (12, 17, 18, 22). However, the molecular basis of the lipid modification of these small GTPases remains largely unknown in this parasite.

In this report, we describe the molecular and biochemical characterization of the α- and β- subunits of FT of *E. histolytica* (*Eh*FT) using recombinant proteins co-expressed in *Escherichia coli*. We also show that only one amebic Ras protein among the many small GTPases tested is farnesylated by *Eh*FT. In addition, we show that the amebic FT exhibits marked resistance to a variety of compounds that are known to inhibit mammalian FT, indicating that the amebic FT possesses distinct biochemical properties from the mammalian FT.

EXPERIMENTAL PROCEDURES

Parasite—Trophozoites of *E. histolytica* strain HM-1:IMSS cl6 (23) were cultured axenically in BI-S-33 medium at 35.5 °C (24).

Chemicals—Recombinant human H-Ras-CVLS (wild type), H-Ras-CVLL (mutant type), FPT inhibitor-I {(*E,E*)-2-[(dihydroxyphosphinyl)-methyl]-3-oxo-3-[(3,7,11-trimethyl-2,6,10-dodecatrienyl)-amino]propanic acid, trisodium salt}, FPT inhibitor-II {(*E,E*)-2-[2-oxo-2-[[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino]ethyl]phosphonic acid, disodium salt}, Gliotoxin, α-hydroxyl farnesylphosphonic acid, and a peptidomimetic inhibitor FTI-276 {*N*-[2-phenyl-4-*N*-[2(R)-amino-3-mercaptopropylamino]benzoyl]-methionine}] were obtained from EMD Biosciences (San Diego, CA). [^{3}H]FPP (16.1 Ci/mmol) and [^{3}H]geranylgeranyl pyrophosphate (23.0 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA), and [^{35}S]GTPγS (1,173 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ). Restriction endonucleases and modifying enzymes were purchased from Takara Biochemical (Tokyo, Japan). The other chemicals and reagents used were from either Sigma-Aldrich Fine Chemicals (Tokyo, Japan) or Wako Pure Chemical Industries (Osaka, Japan) unless otherwise mentioned and of the highest purity available.

cDNA Library of E. histolytica—A trophozoite cDNA library of *E. histolytica* was constructed using the poly(A)$^+$ RNA and λZAP II phage (Stratagene, La Jolla, CA) as described previously (25).

Identification and Cloning of FTα and FTβ of E. histolytica—We designed oligonucleotide primers to amplify FTα and FTβ protein-coding regions from *E. histolytica* by PCR based on a homology search using yeast and mammalian FT in the *E. histolytica* genome data base available at The Institute for Genomic Research (www.tigr.org/tdb/). The sense primer for *EhFTα* was 5′-ATGGAAGAAGACGAAGAAATCACATTTG-3′. Sense and antisense primers for *EhFTβ* were 5′-ATGGAAATTGAAGAAGTAGAAGTAGAAACTGTTAC-3′ and 5′-TTAGAGCGAACGGAAATACTCACAACGCTTATC-3′, respectively. Since we did not find a sequence containing the C terminus of FTα in the data base, we used T7 primer, located downstream of the cloning site of our cDNA library, to amplify the FTα-coding region. PCR was performed using a one-hundredth volume of the cDNA phage lysate as template with the following parameters. An initial step of denaturation at 94 °C for 3 min was followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 2 min. A final step at 72 °C for 10 min was used to complete the extension. The amplified DNA fragments were electrophoresed, purified using a Geneclean II kit (BIO101, La Jolla, CA), and cloned into the SmaI site of pUC18 using a SureClone Ligation Kit (Amersham Biosciences). Nucleotide sequences were confirmed with an ABI Prism BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) on an ABI Prism 310 Genetic Analyzer.

Identification and Cloning of Ras Small G Proteins of E. histolytica—To identify substrates for the amebic FT, we searched for putative Ras homologues in the *E. histolytica* Genome data base via a TBLASTN search using amebic (*Eh*Ras1and *Eh*Ras2) and mammalian Ras as inquiry sequences. We identified 8 previously uncharacterized putative full-length Ras genes. The C terminus of these Ras proteins ended with Phe (four genes), Leu (two), Met (one), or Ala (one). The two latter genes encoding putative Ras proteins containing the CSVM- or CVVA-C terminus (identical to EH02830 and EH01021 in the *E. histolytica* genome data base) were assumed to be good candidates to be farnesylated by the amebic FT, designated *Eh*Ras3 and *Eh*Ras4, respectively, and characterized further. A protein coding region of *Eh*Ras1-4, and *Eh*RacC were amplified by PCR using cDNA as template and appropriate primers based on the sequences in the genome data base.

Sequence Analysis—FTα and FTβ protein sequences from *E. histolytica* and 9 other organisms, and 20 putative Ras and Ras-related proteins of *E. histolytica* were retrieved from the TIGR and the National Center for Biotechnology Information data bases (www.ncbi.nih.gov/) using the BLASTP and TBLASTN algorithms. The protein alignment and phylogenetic analyses were performed with ClustalW version 1.81 (26) using the neighbor-joining (NJ) method (27) with the Blosum matrix created using the ClustalW program (26). Unrooted NJ trees were drawn with TreeView ver.1.6.0 (28). Branch lengths and bootstrap values (1000 replicates) (29) were derived from the NJ analysis.

Construction of a Plasmid to Express Recombinant EhFT—A plasmid containing the protein-coding regions of FTα (without the stop codon), FTβ (with the stop codon), and the ribosome binding sequence (GAGGAGTTTTAACTT) between them were constructed by three rounds of PCR using the recombinant approach (30, 31). Briefly, two sets of initial PCRs were conducted to amplify the FTα and FTβ protein-coding region using a sense primer, 5′-ATGGAAGAAGACGAAGAAATCACATTTG-3′ and an antisense primer, 5′-ATGATTAGTAATTTTTGTTAAATACCAATCCC-3′ (for FTα); a sense primer, 5′-ATGGAAATTGAAGAAGTAGAAGTAGAAACTGTTAC-3′ and an antisense primer, 5′-TAAGAGCGAACGGAAATACTCACAAGCCTTATC-3′ (for FTβ). Two sets of second PCRs were conducted using the respective product of the first reaction as a template. To amplify the FTα protein-coding region (excluding the stop codon), flanked by a BamHI site (italicized) and the ribosome binding site (underlined), a sense primer, 5′-GGA*GGATCC*CATGGAAGAAGACGAAGAAATCACATTTG-3′ (primer 1) and an antisense primer, 5′-AAGTTAAAACTCCTCATGATTAGTAATTTTTGTTAAATACCAATCCC-3′, were used. To amplify the FTβ sequence including the stop codon, flanked by the ribosome binding site (underlined) and a HindIII site (italicized), sense, 5′-GAGGAGTTTTAACTTATGGAAATTGAAGAAGTAGAAGTAGAAACTGTTAC-3′ and antisense, 5′-CCAA*AGCTTT*AAGAGCGAACGGAAATACTCACAAGCCTTATC-3′ (primer 2) were used. The third round of PCR was conduced using a mixture of the products of the second round, and primers 1 and 2. The resulting 2.1-kb PCR product was digested with BamHI and HindIII and ligated into BamHI- and HindIII-double digested pQE31 to construct pEhFTαβ. In pEhFTαβ, the FTα and FTβ protein-coding regions placed in tandem were presumably translationally coupled, facilitating co-expression of these two subunits at similar levels. An N-terminal histidine tag was also engineered in EhFTα to facilitate purification.

Construction of Plasmids to Express Recombinant Small GTPases—A protein-coding region of *Eh*Ras1-4, and *Eh*RacC flanked by additional BamHI and SalI sites (italicized), were amplified by PCR using cDNA as a template and sense and antisense primers: 5′-GGA*GAATCC*CATGACTGCCAATACATATAAATTAGTTATG-3′ and 5′-CC*AGTCGAC*TTAGAACATTATGCATTTCTTTTCTTTCTT-3′ (*Eh*Ras1); 5′-GGA*GAATCC*CATGACTACAAATACTTATAAATTAGTTATGCTTG-3′ and 5′-CCA*GTCGAC*TTATAACAATTCACACTTTGATTTAGAAGG-3′ (*Eh*Ras2); 5′-GGA*GAATCC*CATGAGTTTAAAAAGAATTGTTATGCTTGGA and 5′-CCA*GTCGAC*TTACATAACAGAACATCCAATTTTCTTATA-3′ (*Eh*Ras3); 5′-GGA*GAATCC*CATGAACTCAACAATAAAAAGAATATCTGTT-3′ and 5′-CCA*GTCGAC*TTAAGCAACCACACATGAAGTATTATTCTC-3′ (*Eh*Ras4); 5′-GGA*GAATCC*CATGAGTGAAAAACCCACATCAAT-3′ and 5′-CCA*GTCGAC*TTATAAAAGAGCACACTTTGACCTTTG-3′ (*Eh*RacC), where restriction sites are italicized. PCR products were electrophoresed, purified, and cloned into BamHI- and SalI double-digested pQE31 plasmid to obtain pEhRas1-4 and pEhRacC. The resulting plasmids were designed to contain an N-terminal histidine tag to facilitate purification. Plasmids to express an N-terminal Nus fusion protein of *Eh*Rap2 and a glutathione S-transferase-*Eh*Rab5 or glutathione *S*-transferase-*Eh*Rab7 fusion protein were constructed using pET-43.1a and pGEX4T-1 with appropriate restriction sites included in PCR primers. The characterization of *Eh*Rab5, *Eh*Rab7, and *Eh*Rap2 and details of the construction of these expression plasmids are described elsewhere.

Expression and Purification of Recombinant Proteins—Plasmids constructed as described above were introduced into *E. coli* M15 cells. A 12-ml seed culture was grown overnight at 37 °C in LB medium containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin. The overnight culture was then inoculated into 250 ml of fresh medium containing the antibiotics. The bacteria were grown for 1 h, and then another 4 h after the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside to induce protein expression. The bacteria were harvested by centrifugation at 4,000 × *g* for 20 min, and the pellet was stored at −20 °C until purification. The recombinant proteins were purified according to the manufacturer's instructions. Briefly, the bacterial cells were resus-

pended in cold lysis buffer, phosphate-buffered saline, pH 8.0, containing 10 mM imidazole and 1% lysozyme, sonicated, and centrifuged at 10,000 × *g* for 20 min. The supernatant was applied to a Ni-NTA-agarose column (Qiagen, Hilden, Germany), washed extensively with the wash buffer containing 20 mM imidazole, and eluted with the lysis buffer containing 250 mM imidazole. The recombinant FT proteins were further purified with Q Sepharose Fast Flow (Amersham Biosciences) at a flow rate of 0.5 ml/min as described (32). The purified recombinant FT and Ras proteins were then dialyzed against the enzyme assay buffer described below and 40 mM Tris-HCl, pH 8, containing 90 mM NaCl, 10 mM $MgCl_2$, and 2 mM dithiothreitol and stored with 20 and 50% glycerol, respectively, at −80 °C until use. Protein concentrations were determined by the method of Bradford (33) using Protein Assay CBB solution (Nacalai Tesque, Kyoto, Japan). Bovine serum albumin was used as the protein standard.

Protein Analyses—The expression and purity of recombinant proteins were evaluated by standard SDS-PAGE as described (34). To prepare *E. histolytica* extracts, trophozoites were washed three times with ice-cold phosphate-buffered saline, resuspended at 10^7/ml in phosphate-buffered saline containing a proteinase inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 1 nM trypsin inhibitor, 100 μM *trans*-epoxysuccinyl-L-leucylamino-(4-guanidino)butane, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml *N*-α-p-tosyl-L-lysine chloromethyl ketone hydrochloride, and 1 mM benzamidine hydrochloride), and subjected to 3 cycles of freezing and thawing. After centrifugation at 10,000 × *g* for 10 min, the supernatant was subjected to further analyses.

Enzyme Assays—The enzymatic activity of recombinant FT and the whole lysate of *E. histolytica* trophozoites were assayed for incorporation of [^{3}H]farnesyl pyrophosphate or [^{3}H]geranylgeranyl pyrophosphate into the recombinant small GTPases prepared as described above, human H-Ras-CVLS, or H-Ras-CVLL. The assay was performed essentially as described previously (35) with minor modifications. Briefly, in standard assays, the reaction mixture contained, in a total volume of 50 μl, 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 20 μM $ZnCl_2$, 5 mM dithiothreitol, 0.1% polyethylene glycol 20,000, 187 nM [^{3}H]FPP (3 μCi/ml), or [^{3}H]geranylgeranyl pyrophosphate (3 μCi/ml), and 240 to 750 μM of the purified recombinant FT or 1.9 mg/ml of the *E. histolytica* lysate. The reaction was initiated by the addition of either the recombinant enzyme or cell extracts, run at 30 °C for 20 min, and terminated by the addition of 200 μl of 10% HCl in ethanol. The quenched reactions were allowed to stand at room temperature for 15 min. After the addition of 200 μl of 100% ethanol, the reactions were vacuum-filtered through glass filter GF/C (Whatman, Maidstone, UK) using a Sampling Manifold (Millipore Corp., Bedford, MA). The filters were washed with 4 ml of 100% ethanol and then subjected to scintillation counting (LS 6,000IC, Beckman Coulter, Fullerton, CA). The K_m value was calculated from Lineweaver-Burk plots. FT assays were also conducted in the presence of known FT inhibitors: farnesylpyrophosphate analogues (FPT inhibitor-I, FPT inhibitor-II, Gliotoxin, α-hydroxyl farnesylphosphonic acid) and a peptidomimetic inhibitor (FTI-276) under the condition described above.

Guanine Nucleotide Binding Assays—GTP binding activity was measured using [^{35}S]GTPγS (Amersham Biosciences) and a nitrocellulose filter (Millipore HA filter, Millipore Corporation) as previously described (36).

RESULTS

Features of FTα and FTβ of E. histolytica—Nucleotide sequences of *Eh*FTα and *Eh*FTβ obtained by PCR were identical to sequences available from the genome data base (EH02829 and EH04188, respectively). The putative protein-coding region of *Eh*FTα and *Eh*FTβ, consisting of 894 and 1,125 bps, encodes proteins of 298 and 375 amino acids with a calculated molecular mass of 35.6 and 42.6 kDa and a pI of 5.4 and 5.7, respectively. A search for previously identified domains and motifs (37) using the NCBI Conserved Domain Search revealed that both *Eh*FTα and *Eh*FTβ contained well conserved signature domains shared by other organisms. *Eh*FTα contained one BET4 domain and five "protein prenyltransferase α-subunit repeats"; *Eh*FTβ possessed one CAL1 domain and five "prenyltransferase and squalene oxidase repeats" (Fig. 1). The deduced protein sequences of *Eh*FTα and *Eh*FTβ were aligned with those from other organisms using the ClustalW program (Fig. 1). Both *Eh*FTα and *Eh*FTβ were the smallest in size, and lack any recognizable secretory signal sequence, an organelle targeting signal, and any domain implicated in membrane association including the transmembrane domain and myristylation signal. *Eh*FTα and *Eh*FTβ also lack the N-terminal extension of 15–58 amino acids found in these subunits from other organisms. *Eh*FTα showed 29, 30, 27, and 24% positional identity with FTα of human, *Arabidopsis thaliana, Saccharomyces cerevisiae*, and *T. brucei*, respectively; *Eh*FTβ revealed 36, 35, 28, and 31% positional identity with the FTβ of these organisms, respectively. All the residues implicated to be essential for catalysis (Fig. 1, *A* and *B*) (39–41) are conserved in both *Eh*FTα and *Eh*FTβ.

Phylogenetic Analysis of EhFTα and FTβ—Phylogenetic trees of *Eh*FTα and *Eh*FTβ were constructed (Fig. 2). Neither the α- nor β-subunit of the amebic FT showed statistically significant (*i.e.* supported with high bootstrap values) affinity to those from other organisms while trypanosomal, mammalian, and plant proteins encoding α- and β-subunits formed well supported independent clades, representing distinct groups. These results were compatible with the notion that both subunits of *Eh*FT developed independently from other eukaryotes, suggestive of the presence of unique biochemical properties of *Eh*FT (see below).

Expression of EhFT in E. coli—A complex consisting of *Eh*FTα and *Eh*FTβ was expressed and purified as described under "Experimental Procedures." Purified proteins revealed two major proteins with an equal intensity of an apparent molecular mass of 38 and 43 kDa (Fig. 3), which likely correspond to *Eh*FTα and *Eh*FTβ, respectively. The apparent molecular mass of α- and β-subunits agreed well with a theoretical value of 37.6 kDa with the N- and C-terminal addition of MRGSHHHHHHTDP and EEF, respectively, for the recombinant *Eh*FTα and 42.6 kDa for *Eh*FTβ. When histidine-tagged *Eh*FTα or *Eh*FTβ were expressed separately, the apparent molecular mass of these proteins agreed well with their identity (data not shown). Densitometric quantitation of these two bands also supported that they contain an equal number of protein molecules (data not shown). Gel filtration chromatography of the recombinant *Eh*FT using Sephacryl S-300 revealed a molecular mass of about 80 kDa (data not shown). Thus, the recombinant *Eh*FTα and *Eh*FTβ were present as a stable dimer during the process of purification by Ni-NTA agarose and Q Sepharose Fast Flow chromatography (Fig. 3). After the purification, recombinant *Eh*FT was estimated to be >95% pure by densitometric quantitation (data not shown), and further utilized for enzymatic assays.

Demonstration of FT Activity of the Recombinant EhFT against Human Ras Proteins—When assayed for incorporation of [^{3}H]farnesyl pyrophosphate, the recombinant *Eh*FT showed FT activity [1.03 ± 0.012 nmol of FPP/mg of protein (mean ± S.E.)] against human recombinant wild-type H-Ras-CVLS, whereas it showed ~20-fold less activity against mutant H-Ras-CVLL (0.05 ± 0.01 nmol of FPP/mg of protein) (Fig. 4), which was previously shown to be predominantly geranylgeranylated by human GGT-I. The addition of EDTA (10 mM) to the reaction mixtures completely abolished the enzymatic activity of the recombinant *Eh*FT (data not shown), suggesting, as shown for mammalian and yeast FT, that *Eh*FT also requires Zn^{2+} and Mg^{2+} for its activity.

Identification and Phylogenetic Analysis of the Novel Ras Proteins in E. histolytica—To identify the protein substrates of *Eh*FT in the parasite, we searched for putative Ras proteins in the genome data base based on homology to the *Eh*Ras1 and human H-Ras. In addition to *Eh*Ras1, *Eh*Ras2, *Eh*Rap1, and *Eh*Rap2, which have already been reported (12), we found 8 additional putative ras proteins previously uncharacterized, with the C-terminal *CaaX* motif. Of these 8 proteins, 6 possess

FIG. 1. **Alignment of the deduced amino acid sequences of α- (*A*) and β- (*B*) subunits of protein farnesyltransferase of *E. histolytica* with those from other organisms.** *Eh*, *Entamoeba histolytica*; *Hs*, *Homo sapiens*; *At*, *A. thaliana*; *Sc*, *S. cerevisiae*; *Tb*, *T. brucei*. *Asterisks* (*) under the alignments indicate identical amino acid residues and *dots* (.) indicate conserved amino acid substitutions. BET4 (in *A*) and CAL1 (in *B*) domains detected by the NCBI Conserved Domain Search are *dotted-underlined* (partially *solid underlined*). The prenyltransferase α-subunit repeats and the prenyltransferase/squalene oxidase repeats, also detected by the search, are indicated by *solid underline* in *A* and *B*, respectively. The regions corresponding to the motif P*X*N*YXX*WYR (37), previously found in the turns connecting two helices of the coiled-coil, in FTα and a glycine-rich motif GG*F*/*YG*/*AXX*P (37), corresponding to the loop regions that connect the C termini of the peripheral helices with the N termini of the core helices in the barrel (38) in FTβ are *boxed*. All amino acids implicated in catalysis by crystallographic and mutational studies of mammalian FTs (38–41) are shaded. Aromatic amino acids located in the hydrophobic cleft at the center of the α-α barrel implicated in the interaction with the farnesyl residue are marked with *open circles* (38). Arg^{202} implicated in the binding of the essential C-terminal carboxylate of the protein substrate is marked with a *filled circle* (39). Amino acids implicated in the coordination of zinc are marked with *open squares* (40). Amino acids implicated in the binding and utilization of FPP, shown by a mutational analysis (39) are marked with *filled squares*. N-terminal extensions absent in *Eh*FTα and *Eh*FTβ are also *boxed*. DDBJ/EMBL/GenBank™ accession numbers are given in Fig. 2.

Phe or Leu (4 with Phe, 2 with Leu) at the C terminus, while 1 each has Met or Ala. We tentatively designated proteins possessing Met or Ala as *Eh*Ras3 or *Eh*Ras4, and the other proteins as *Eh*Ras5–10. The nucleotide sequence of the *Eh*Ras1 cDNA we cloned was identical to that previously reported; the nucleotide sequence of our *Eh*Ras2 cDNA differed at one nucleotide (A368G) from the sequence previously reported (12), resulting in a Y123C substitution. *Eh*Ras3 and 4 consisted of 210 and 182 amino acids with a calculated molecular mass of 23.9 and 20.6 kDa and a pI of 5.5 and 5.8, respectively. The ClustalW multiple alignment showed that *Eh*Ras3 and 4, together with the previously identified *Eh*Ras1, *Eh*Ras2, *Eh*Rap1, and *Eh*Rap2, share conserved GTP binding consensus sequences (42) and also, at a moderate level, the effector binding region (42) (Fig. 5*A*).

Percent identity among the *Eh*Ras1-4 and *Eh*Rap1-2 (Fig. 5*B*) also indicates that *Eh*Ras3 is, together with *Eh*Ras5 and *Eh*Ras6 (*Eh*Ras5 *versus Eh*Ras1-2, 62–66%; *Eh*Ras6 *versus Eh*Ras1-2, 39–41%; *Eh*Ras5 and *Eh*Ras6 were not studied further in the present work) closely associated with *Eh*Ras1

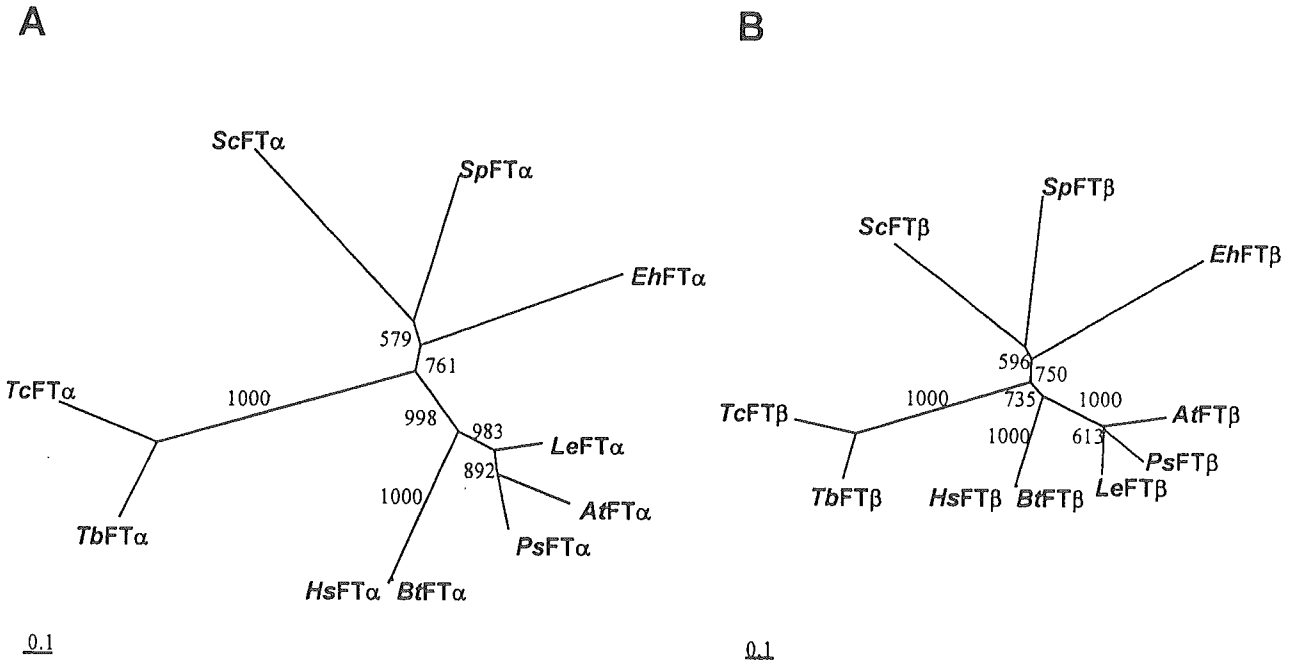

FIG. 2. **Phylogenetic trees of FTα (*A*) and FTβ (*B*).** Only FTα and FTβ protein sequences that were previously characterized enzymologically were included. The *trees* were constructed by Neighbor-joining distance analysis using the ClustalW and TreeView programs. *Line lengths* indicate distances between nodes. The *bar* represents a distance of 0.1 amino acid changes per site. Bootstrap values for 1,000 replicates are shown at nodes. Abbreviations are: *Eh*, *E. histolytica* (DDBJ/EMBL/GenBank™ number of FTα and FTβ, AB083372, AB083373); *Sc*, *S. cerevisiae* (P29703, P22007); *Sp*, *Schizosaccharomyces pombe* (O60052, O13782); *Tb*, *T. brucei* (AAF73919, AAF73920); *Tc*, *T. cruzi* (AAL69904, AAL69905); *At*, *A. thaliana* (Q9LX33, AAF74564); *Ps*, *Pisum sativum* (O24304, Q04903); *Le*, *Lycopersicon esculentum* (P93277, AAC49666); *Bt*, *Bos taurus* (NP_803464, P49355). *Hs*, *Homo sapiens* (NP_002018, NP_002019).

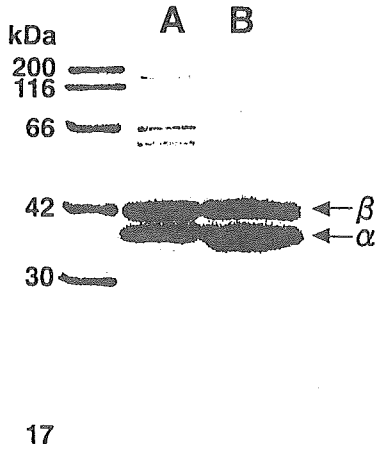

FIG. 3. **SDS-PAGE analysis of the purified recombinant FT of *E. histolytica*.** Both *Eh*FT subunits were coexpressed in *E. coli*, copurified on Ni-NTA agarose (*lane A*), or further purified with anion exchange Q Sepharose Fast Flow (*lane B*). Samples were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

and *Eh*Ras2, showing 48–51% identities, whereas *Eh*Ras4 showed only 26–30% identities to *Eh*Ras1, *Eh*Ras2, *Eh*Rap1, and *Eh*Rap2. Phylogenetic reconstructions (Fig. 6) confirmed the results of the protein alignment: both *Eh*Ras3 and *Eh*Ras4, together with *Eh*Ras5 and *Eh*Ras6, represent new members of the Ras/Rap family. Rac and Rab proteins were categorized to independent clades, whose association was well supported by moderate to high bootstrap values (only representative Rac and Rab proteins were included in this analysis).

Identification of EhRas4 as a Substrate of Eh FT—We tested substrate specificity of *Eh*FT toward *Eh*Ras1-4. We chose *Eh*Ras3-4, together with *Eh*Ras1-2, as possible candidates for *Eh*FT substrates because it was previously shown that mammalian and yeast small GTPases with a C-terminal Ser, Met, Gln, Cys, or Ala have a tendency to be farnesylated whereas those containing Phe or Leu at the C-terminal end tend to be geranylgeranylated (4). The recombinant *Eh*FT showed farnesyltransferase activity toward *Eh*Ras4 (1.03 ± 0.005 nmol of FPP/mg of protein), which was comparable to the activity toward human H-Ras-CVLS (Fig. 4). In contrast, *Eh*FT showed no detectable or only minimal activity toward *Eh*Ras1-3. We also tested if *Eh*Rab, *Eh*Rac, or *Eh*Rap are farnesylated by *Eh*FT. The recombinant *Eh*FT did not transfer farnesyl to *Eh*Rab7, *Eh*RacC, or *Eh*Rap2 (data not shown). Furthermore, the recombinant *Eh*FT did not utilize geranylgeranyl pyrophosphate as a isoprenyl donor to transfer the geranylgeranyl residue to *Eh*Ras1, *Eh*Ras2, *Eh*Ras3, *Eh*Ras4, *Eh*Rap2, *Eh*RacC, or *Eh*Rab7 (data not shown). To confirm that *Eh*Ras3 and 4 are capable of binding GTP, a GTP binding assay was conducted. Both *Eh*Ras3 and 4 showed comparable GTP-binding activity to *Eh*Ras2 and *Eh*Rab7 (data not shown). We also assayed for the FT activity in the whole lysate of the *E. histolytica* trophozoites. Among the 2 human H-Ras and 4 *E. histolytica* Ras homologues described above, FT activity was detected only against human H-Ras-CVLS and *Eh*Ras4 in the whole lysate (data not shown), which excluded the possibility that some other *Eh*FT protein (or proteins) exists to farnesylate these small GTPase in the amebic lysate.

Kinetic Properties of EhFT—Lineweaver-Burk plots showed the K_m of recombinant *Eh*FT for *Eh*Ras4 to be 5.13 ± 0.02 μM (plots not shown), significantly higher than that of bovine FT, the K_m of which for Ras-CVLS and Ras-CVIM is 0.63 ± 0.05